(br s, 1H, CONH), 9.81 (br s, 1H, CONH), 7.75 (dd, 1H, aromatic), 7.70 (s, 1H, aromatic), 6.93 (td, 1H, aromatic), 6.84 (m, 1H, aromatic), 3.63 (m, 2H, CH_2), 3.29 (m, 2H, CH_2), 3.14 (m, 4H, 2xCH_2), 2.47 (s, 1H, CH_3), 2.45 (s, 3H, CH_3), 1.64 (t, 6H, 2xCH_3). MS *m/z* 415 [M+1].

Example 190

5-[5-fluoro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(pyridin-1-yl)ethyl]-amide.

5-[5-Fluoro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (120 mg, 0.4 mmol) was shaken with EDC, HCl (96 mg, 0.5 mmol), anhydrous 1-hydroxy-benztriazole (68 mg, 0.5 mmol), and 2-(2-aminoethylpyridine purchased from Aldrich in anhydrous DMF (3 mL) for 2-3 days at room temperature. The reaction mixture was diluted with 1M NaHCO3 (1.5 ml), then with 8 ml of water. The precipitated crude product was collected by filtration, washed with water, dried and purified by crystallization or chromatography to give 5-[5-fluoro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(pyridin-1-yl)-ethyl]amide.

Example 189

5-[5-Chloro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(pyridin-1-yl)ethyl]amide.

Proceeding as described in previous example but substituting 5-[5-fluoro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid with 5-[5-chloro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (127 mg) provided 5-[5-chloro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(pyridin-1-yl)ethyl]amide.

Example 192

5-[5-Bromo-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(pyridin-1-yl)ethyl]amide.

Proceeding as described in Example 190 above but substituting 5-[5-fluoro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid with 5-[5-bromo-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (145 mg) provided 5-[5-bromo-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(pyridin-1-yl)ethyl]amide.

Example 191

5-[2-Oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(pyridin-1-yl)ethyl]amide

Proceeding as described in Example 190 above but substituting 5-[5-fluoro-2-oxo-1,2-dihydro-indol-(3Z)-ylidene-methyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid with 5-[2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (113 mg) provided 5-[2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(pyridin-1-yl)ethyl]amide.

Example 203

5-[5-Cyano-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(pyridin-1-yl)ethyl]amide

Proceeding as described in Example 190 above but substituting 5-[5-fluoro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid with 5-[5-cyano-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (123 mg) provided 5-[5-cyano-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(pyridin-1-yl)ethyl]amide.

Examples 142, 186, 187, 188 and 204

Proceeding as described in Examples 190, 189, 191, 192,

and 203 above but substituting 2-(2-aminoethyl)pyridine with 1-(2-aminoethyl)pyrrolidine, purchased from Aldrich Chemical Company, Inc. provided the desired compounds.

Examples 143-147

Proceeding as described in Examples 190, 189, 191, 192, and 203 above but substituting 2-(2-aminoethyl)pyridine with 1-(2-aminoethyl)imidazolin-2-one (prepared by heating dimethyl carbonate with bis(2-aminoethyl) amine (2 equivalents) in a sealed flask to 150 °C for 30 min., following the procedure described in U.S.Patent 2613212 (1950), to Rohm & Haas Co. The crude product was purified on silica using an eluent mixture chloroform-methanol-aqueous ammonia 80:25:2) provided the desired compounds.

Examples 148-151 and 184

Proceeding as described in Examples 190, 189, 191, 192, and 203 above but substituting 2-(2-aminoethyl)pyridine with 4-(2-aminoethyl)piperazine-1-acetic acid ethyl ester (prepared as follows: Piperazine-1-acetic acid ethyl ester (11.22 g) was treated with iodoacetonitrile (5. 0 mL) in the presence of potassium carbonate (6.9 g) in ethyl acetate (260 mL) at 0 °C. After complete iodoacetonitrile addition (45 min), the reaction mixture was subsequently stirred at room temperature for 11 hours. The reaction mixture was filtered and the filtrates evaporated. The residue was hydrogenated in a presence of cobalt boride (prepared from CoCl2 and sodium borohydride) at room temperature at 50 psi for 2 days in ethanol. Filtration, evaporation and chromatographic purification using an eluent mixture chloroform-methanol-aqueous ammonia 80:25:2 provided the desired amine (3.306 g) as a pale yellow oil) provided the desired compounds.

Example 152-153

Proceeding as described in Examples 190, 189, 191, 192, and 203 above but substituting 2-(2-aminoethyl)pyridine with 2-[(2-aminoethylamino)]acetonitrile (prepared as follows: A solution of iodoacetonitrile (50 mmol) in ethyl alcohol (80 ml) was added to a solution of ethylene diamine (150 ml) in

ethyl alcohol (60 ml) at 0 °C over a period of 30 minutes. The stirring was continued for another 1 hr at 0 °C, then at room temperature for 14 hours. 55 mmol of potassium carbonate was added, stirred for 30 minutes, filtered and the filtrate was concentrated at room temperature. The residue was purified on silica using an eluent mixture chloroform-methanol-aqueous ammonia 80:15:1.5 to give 2-[(2-aminoethylamino)]-acetonitrile (3.550 g) which was used immediately) provided the desired compounds.

Example 154-158

Proceeding as described in Examples 190, 189, 191, 192, and 203 above but substituting 2-(2-aminoethyl)pyridine with 1-(3-aminopropyl)-azepin-2-one (prepared according to the procedure in Kraft A.: J. Chem. Soc. Perkin Trans. 1, 6, 1999, 705-14, except that the hydrolysis of DBU was performed at 145 °C neat in a presence of lithium hydroxide (1 hr, 5 ml of DBU, 2 ml of water, 420 mg of lithium hydroxyde hydrate). Purification of the crude product on silica using an eluent mixture chloroform-methanol-aqueous ammonia 80:40:4 provided 1-(3-aminopropyl)azepin-2-one (4.973g, 87 % yield)) provide the desired compounds.

Examples 133-135, 159 and 200

Proceeding as described in Examples 190, 189, 191, 192, and 203 above but substituting 2-(2-aminoethyl)pyridine with N-acetyl ethylene diamine, (prepared by heating a mixture of ethyl acetate with ethylene diamine (1.5 equivalents) to 160 °C for 1 hr in a sealed vessel. The vacuum distillation provided the desired product in 56% yield. N-acetylethylene diamine is also available from Aldrich) provide the desired compounds.

Examples 146-140

Proceeding as described in Examples 190, 189, 191, 192, and 203 above but substituting 2-(2-aminoethyl)pyridine with 1-(3-aminopropyl)-tetrahydro-pyrimidin-2-one (prepared in the same way as 1-(3-aminopropyl)-azepin-2-one according to the procedure in Kraft A.: J. Chem. Soc. Perkin Trans. 1, 6, 1999, 705-14: Briefly, 1,3,4,6,7,8-hexahydro-2H-pyrimido[1,2-

a]pyrimidine (4.939 g), lithium hydroxyde hydrate (918 mg) and 2 ml of water was heated without a solvent in a sealed vessel to 145 °C for 1hr. The crude product was purified on a column of silica in chloroform-methanol-aqueous ammonia 80:40:4 to give pure amine (5.265g, 94% yield).

Examples 141, 160-162 and 185

Proceeding as described in Examples 190, 189, 191, 192, and 203 above but substituting 2-(2-aminoethyl)pyridine with 1-(2-aminoethyl)-piperazine-2-one (prepared as follows: Neat tert-butyldiphenylsilyl chloride (25 mL, 97.7 mmol) was added dropwise into a solution of DBU (19.5 ml, 130 mmol) and bis(2-aminoethyl)amine (4.32 mL, 40 mmol) in anhydrous dimethyl acetamide (80 mL) at room temperature upon cooling on water bath within 5 minutes. The mixture was stirred for 5 hours. Bromoacetic acid ethyl ester (6.70 mL, 60 mmol) was added neat upon cooling to room temperature. The reaction was stirred for 25 minutes, then evaporated on high vacuum. The residue was dissolved in methanol (200 ml), $KHCO_3$ (10g) and KF (12 g, 200 mmol) were added and the mixture was stirred at 60 °C for 5 hours. 10g of Na_2CO_3 was added, stirred for 10 minutes, cooled and filtered. The filtrates were evaporated. The residue was extracted with hexanes (2 times 250 ml). The hexane-insoluble material was dissolved in ethanol (60ml), filtered and evaporated. The residue was purified ona column of silica in chloroform-methanol-aqueous ammonia 80:40:4 to give pure amine (4.245g,74% yield)) provided the desired compounds.

Examples 163-167

Proceeding as described in Examples 190, 189, 191, 192, and 203 above but substituting 2-(2-aminoethyl)pyridine with 3-[(2-aminoethyl)amino]propionitrile (prepared from ethylene diamine (150 mmol) and acrylonitrile (50 mmol) in THF at room temperature, as described in Israel, M. et al: J. Med Chem. 7, 1964, 710-16., provided the desired amine (4.294 g)) provided the desired compounds.

Example 168

5-(5-Fluoro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid [2-(4-methylpiperazin-1-yl)-ethyl]-amide

To a stirred yellow muddy mixture of 5-[5-fluoro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (90 mg), DMF (0.8 mL) and TEA (0.084 mL) in a 20 mL reaction tube, was added BOP reagent (199 mg). The mixture became clear in 5 min. 2-(4-Methylpiperazin-1-yl)ethylamine[1] (51 mg) was added into the clear mixture. The resulting solution was stirred at room temperature over night. Yellow solid products precipitated from the reaction system. Thin layer chromatography (10% methanol in methylene chloride) showed that all the starting material had been converted into the product. The solid was isolated by vacuum filtration and washed once with ethanol (1 mL). The solid was sonicated in diethyl ether (2 mL) for 20 min and collected by vacuum filtration. After drying under vacuum, 5-(5-Fluoro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (4-methylpiperazin-1-yl-ethyl)-amide (79 mg, 62% yield) was obtained.

^{1}H NMR (DMSO-d_6) δ 2.13 (s, 3H, CH_3), 2.40, 2.42 (2xs, 6H, 2x CH_3), 2.41 (m, 2H, CH_2), 2.47 (m, 8H, 4xCH_2), 3.30 (m, 2H, CH_2), 6.82 (dd, J=4.5, 8.7Hz, 1H), 6.91(td, ^{2}J=2.4, ^{3}J=8.8Hz, 1H), 7.43 (t, J=5.6Hz, 1H), 7.70 (s, 1H), 7.75 (dd, J=2.8, 9.6Hz, 1H) (aromatic and vinyl), 10.88 (s, 1H, CONH), 13.67 (s, 1H, NH). LC-MS (m/z) 424.4 (M-1).

Example 169

5-(5-Chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (4-methylpiperazin-1-yl-ethyl)-amide

Following the procedure in Example 168 above but substituting 5-[5-fluoro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid with 5-[5-chloro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (95 mg, 0.3 mmol) gave 5-(5-chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,4-

dimethyl-1H-pyrrole-3-carboxylic acid (4-methylpiperazin-1-yl-ethyl)-amide (76 mg, 58%).

^{1}H NMR (DMSO-d$_6$) δ 2.13 (s, 3H, CH_3), 2.41, 2.42 (2xs, 6H, 2x CH_3), 2.42 (m, 2H, CH_2), 2.48 (m, 8H, 4xCH_2), 3.30 (m, 2H, CH_2), 6.84 (d, J=8.0Hz, 1H), 7.11 (dd, J=2.0, 8.0Hz,1H), 7.44 (t, J=5.6Hz, 1H), 7.76 (s, 1H), 7.97 (d, J=2.0Hz, 1H) (aromatic and vinyl), 10.98 (s, 1H, CONH), 13.62 (s, 1H, NH). LC-MS (m/z) 440.2 (M-1).

Example 170

5-(5-Bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (4-methylpiperazin-1-yl-ethyl)-amide

Following the procedure described in Example 168, but substituting 5-(5-chloro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid with 5-(5-bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid gave 5-(5-bromo-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (4-methylpiperazin-1-yl-ethyl)-amide (39 mg, 54%) was obtained from SU011670 (54 mg, 0.15 mmol). ^{1}H NMR (DMSO-d$_6$) δ 2.14 (s, 3H, CH_3), 2.41, 2.42 (2xs, 6H, 2x CH_3), 2.42 (m, 2H, CH_2), 2.48 (m, 8H, 4xCH_2), 3.31 (m, 2H, CH_2), 6.80 (d, J=8.0Hz, 1H), 7.23 (dd, J=2.0, 8.0Hz, 1H), 7.44 (t, J=5.6Hz, 1H), 7.76 (s, 1H), 8.09 (d, J=2.0Hz, 1H) (aromatic and vinyl), 10.99 (s, 1H, CONH), 13.61 (s, 1H, NH). LC-MS (m/z) 486.6 (M).

Example 172

5-(2-Oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (4-methylpiperazin-1-yl-ethyl)-amide

Following the procedure described in Example 168 above but substituting 5-(5-fluoro-2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid SU014900 with 5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid gave 5-(2-oxo-1,2-dihydro-indol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (4-methylpiperazin-1-yl-ethyl)-amide, SU014903

(136 mg, 84%) was obtained from SU012120 (112.8 mg, 0.4 mmol). ^{1}H-NMR (DMSO-d_6) δ 2.13 (s, 3H, CH_3), 2.39, 2.42 (2xs, 6H, 2xCH_3), 2.42 (m, 2H, CH_2), 2.48 (m, 8H, 4xCH_2), 3.30 (t, 2H, CH_2), 6.86 (d, J=8.0Hz, 1H), 6.96 (t, J=7.4 Hz, 1H), 7.10 (t, J=7.8Hz, 1H), 7.41 (t, J=5.4Hz, 1H), 7.62 (s, 1H), 7.76 (d, J=7.6Hz, 1H) (aromatic and vinyl), 10.88 (s, 1H, CONH), 13.61 (s, 1H, NH). LC-MS (m/z) 406.6 (M-1).

Example 171

5-[2-Oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(3,5-dimethylpiperazin-1-yl)ethyl)amide

To a stirred yellow muddy mixture of 5-[2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (112.8 mg, 0.4 mmol), DMF (0.5 mL) and triethylamine (0.111 mL) in a 20 mL reaction tube, was added BOP reagent (265 mg). The mixture became clear in 5 min. 2-(2,6-dimethylpiperazin-1-yl)ethylamine (68.6 mg) (see., Tapia, L. Alonso-Cires, P. Lopez-Tudanca, R. Mosquera, L. Labeaga, A. Innerarity, A. Orjales, J. Med. Chem., 1999, 42, 2870-2880) was added into the clear mixture. The resulting solution was stirred at room temperature over night. Thin layer chromatography (10% methanol in methylene chloride) showed that all the starting material had been converted into the product. The reaction mixture was evaporated to dryness and then purified by flash chromatography (CH_2Cl_2/CH_3OH=20/1-15/1) followed by recrystalization to give 5-[2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(3,5-dimethylpiperazin-1-yl)ethyl)amide (83 mg, 50% yield).

^{1}H NMR (DMSO-d_6) δ 1.15, 1.16 (2xs, 6H, 2xCH_3), 1.95 (t, J=11.6Hz, 2H, CH_2), 2.41, 2.47 (2xs, 6H, 2xCH_3), 2.50 (m, 2H, CH_2), 3.03 (d, J=10Hz, 2H), 3.19 (m, 2H), 3.30 (m, 2H, CH_2), 6.86 (d, J=8.0Hz, 1H), 6.97 (t, J=7.2 Hz, 1H), 7.11 (t, J=7.8Hz, 1H), 7.48 (t, J=5.6 Hz, 1H), 7.61 (s, 1H), 7.75 (d, J=7.6 Hz, 1H) (aromatic and vinyl), 10.88 (s, 1H, CONH), 13.62 (s, 1H, NH). LC-MS (m/z) 422.2 (M+1).

Example 173

5-[5-Fluoro-2-oxo-1,2-dihydro -indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(3,5-dimethylpiperazin-1-yl)ethyl)amide

Following the procedure described in Example 168 above the desired compound was obtained (60 mg, 0.2 mmol).
^{1}H NMR (DMSO-d_6) δ 0.891, 0.907 (2xs, 6H, 2xCH_3), 1.49 (t, J=10.4Hz, 2H), 2.40, 2.42 (2xs, 6H, 2x CH_3), 2.41 (m, 2H, CH_2), 2.74 (m, 4H), 3.30 (m, 2H), 6.82 (dd, J=4.5, 8.7Hz, 1H), 6.90 (td, ^2J=2.4, ^3J=8.4Hz, 1H), 7.42 (t, J=5.6Hz, 1H), 7.70 (s, 1H), 7.74 (dd, J=4.6, 8.4Hz, 1H) (aromatic and vinyl), 10.88 (s, 1H, CONH), 13.65 (s, 1H, NH). LC-MS (m/z) 438.4 (M-1).

Example 174

5-[5-Chloro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(3,5-dimethylpiperazin-1-yl)ethyl)amide

Following the procedure for Example 171 above the desired compound (31.2 mg, 34%) was obtained from 5-[5-chloro-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (63 mg, 0.2 mmol).
^{1}H NMR (DMSO-d_6) δ 1.15, 1.16 (2xs, 6H, 2xCH_3), 1.95 (t, J=11.6Hz, 2H, CH_2), 2.40, 2.42 (2xs, 6H, 2xCH_3), 2.50 (m, 2H, CH_2), 3.03 (d, J=11.2Hz, 2H), 3.19 (m, 2H), 3.30 (m, 2H, CH_2), 6.85 (d, J=8.4Hz, 1H), 7.11 (dd, J=2.0, 8.0Hz,1H), 7.52 (t, J=5.6Hz, 1H), 7.76 (s, 1H), 7.97 (d, J=2.0Hz, 1H) (aromatic and vinyl), 10.99 (s, 1H, CONH), 13.63 (s, 1H, NH). LC-MS (m/z) 456.2 (M+1).

Example 175

5-[5-Bromo-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid [2-(3,5-dimethylpiperazin-1-yl)ethyl)amide

Following the procedure described in Example 171 the desired compound (40 mg, 40%) was obtained from 5-[5-bromo-2-oxo-1,2-dihydro-indol-(3Z)-ylidenemethyl]-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (74 mg, 0.2 mmol).
^{1}H NMR (DMSO-d_6) δ 1.15, 1.16 (2xs, 6H, 2xCH_3), 1.95 (t,

J=11.6Hz, 2H, CH_2), 2.40, 2.42 (2xs, 6H, 2xCH_3), 2.50 (m, 2H, CH_2), 3.03 (d, J=10.4Hz, 2H), 3.19 (m, 2H), 3.30 (m, 2H, CH_2), 6.81 (d, J=8.4Hz, 1H), 7.24 (dd, J=2.0, 8.4Hz, 1H), 7.51 (t, J=5.6Hz, 1H), 7.76 (s, 1H), 8.10 (d, J=2.0Hz, 1H) (aromatic and vinyl), 10.99 (s, 1H, CONH), 13.62 (s, 1H, NH). LC-MS (m/z) 498.4 (M-1).

Biological Examples

The following assays are employed to find those compounds demonstrating the optimal degree of the desired activity.

A. Assay Procedures.

The following assays may be used to determine the level of activity and effect of the different compounds of the present invention on one or more of the PKs. Similar assays can be designed along the same lines for any PK using techniques well known in the art.

Several of the assays described herein are performed in an ELISA (Enzyme-Linked Immunosorbent Sandwich Assay) format (Voller, *et al.*, 1980, "Enzyme-Linked Immunosorbent Assay," Manual of Clinical Immunology, 2d ed., Rose and Friedman, Am. Soc. Of Microbiology, Washington, D.C., pp. 359-371). The general procedure is as follows: a compound is introduced to cells expressing the test kinase, either naturally or recombinantly, for a selected period of time after which, if the test kinase is a receptor, a ligand known to activate the receptor is added. The cells are lysed and the lysate is transferred to the wells of an ELISA plate previously coated with a specific antibody recognizing the substrate of the enzymatic phosphorylation reaction. Non-substrate components of the cell lysate are washed away and the amount of phosphorylation on the substrate is detected with an antibody specifically recognizing phosphotyrosine compared with control cells that were not contacted with a test compound.

The presently preferred protocols for conducting the ELISA experiments for specific PKs is provided below. However, adaptation of these protocols for determining the

activity of compounds against other RTKs, as well as for CTKs and STKs, is well within the scope of knowledge of those skilled in the art. Other assays described herein measure the amount of DNA made in response to activation of a test kinase, which is a general measure of a proliferative response. The general procedure for this assay is as follows: a compound is introduced to cells expressing the test kinase, either naturally or recombinantly, for a selected period of time after which, if the test kinase is a receptor, a ligand known to activate the receptor is added. After incubation at least overnight, a DNA labeling reagent such as 5-bromodeoxyuridine (BrdU) or H^3-thymidine is added. The amount of labeled DNA is detected with either an anti-BrdU antibody or by measuring radioactivity and is compared to control cells not contacted with a test compound.

GST-FLK-1 BIOASSAY

This assay analyzes the tyrosine kinase activity of GST-Flk1 on poly(glu,tyr) peptides.

Materials and Reagents:

1. Corning 96-well ELISA plates (Corning Catalog No. 5805-96).
2. poly(glu,tyr) 4:1, lyophilizate (Sigma Catalog # P0275).
3. Preparation of poly(glu,tyr)(pEY) coated assay plates: Coat 2 ug/well of poly(glu,tyr)(pEY) in 100 ul PBS, hold at room temperature for 2 hours or at 4^0C overnight. Cover plates well to prevent evaporation.
4. PBS Buffer: for 1 L, mix 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g KCl and 8 g NaCl in approx. 900ml dH_2O. When all reagents have dissolved, adjust the pH to 7.2 with HCl. Bring total volume to 1 L with dH_2O.
5. PBST Buffer: to 1 L of PBS Buffer, add 1.0 ml Tween-20.
6. TBB - Blocking Buffer: for 1 L, mix 1.21 g TRIS, 8.77 g NaCl, 1 ml TWEEN-20 in approximately 900 ml

dH_2O. Adjust pH to 7.2 with HCl. Add 10 g BSA, stir to dissolve. Bring total volume to 1 L with dH_2O. Filter to remove particulate matter.

7. 1% BSA in PBS: To make a 1x working solution, add 10 g BSA to approx. 990 ml PBS buffer, stir to dissolve. Adjust total volume to 1 L with PBS buffer, filter to remove particulate matter.
8. 50 mM Hepes pH 7.5.
9. GST-Flk1cd purified from sf9 recombinant baculovirus transformation (SUGEN, Inc.).
10. 4% DMSO in dH_2O.
11. 10 mM ATP in dH_2O.
12. 40 mM $MnCl_2$
13. Kinase Dilution Buffer (KDB): mix 10 ml Hepes (pH 7.5), 1 ml 5M NaCl, 40 μL 100 mM sodium orthovanadate and 0.4 ml of 5% BSA in dH_2O with 88.56 ml dH_2O.
14. NUNC 96-well V bottom polypropylene plates, Applied Scientific Catalog # AS-72092
15. EDTA: mix 14.12 g ethylenediaminetetraacetic acid (EDTA) to approx. 70 ml dH_2O. Add 10 N NaOH until EDTA dissolves. Adjust pH to 8.0. Adjust total volume to 100 ml with dH_2O.
16. 1^0 Antibody Dilution Buffer: mix 10 ml of 5% BSA in PBS buffer with 89.5 ml TBST.
17. Anti-phosphotyrosine monoclonal antibody conjugated to horseradish peroxidase (PY99 HRP, Santa Cruz Biotech).
18. 2,2'-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS, Moss, Cat. No. ABST).
19. 10% SDS.

<u>Procedure</u>:

1. Coat Corning 96-well ELISA plates with 2 μg of polyEY peptide in sterile PBS as described in step 3 of Materials and Reagents.
2. Remove unbound liquid from wells by inverting plate.

Wash once with TBST. Pat the plate on a paper towel to remove excess liquid.

3. Add 100 µl of 1% BSA in PBS to each well. Incubate, with shaking, for 1 hr. at room temperature.
4. Repeat step 2.
5. Soak wells with 50 mM HEPES (pH7.5) (150 µl/well).
6. Dilute test compound with dH_2O/4% DMSO to 4 times the desired final assay concentration in 96-well polypropylene plates.
7. Add 25 µl diluted test compound to ELISA plate. In control wells, place 25 µl of dH_2O/4% DMSO.
8. Add 25 µl of 40 mM $MnCl_2$ with 4x ATP (2 µM) to each well.
9. Add 25 µl 0.5M EDTA to negative control wells.
10. Dilute GST-Flk1 to 0.005 µg(5 ng)/well with KDB.
11. Add 50 µl of diluted enzyme to each well.
12. Incubate, with shaking, for 15 minutes at room temperature.
13. Stop reaction by adding 50 µl of 250 mM EDTA (pH 8.0).
14. Wash 3X with TBST and pat plate on paper towel to remove excess liquid.
15. Add 100 µl per well anti-phosphotyrosine HRP conjugate, 1:5,000 dilution in antibody dilution buffer. Incubate, with shaking, for 90 min. at room temperature.
16. Wash as in step 14.
17. Add 100 µl of room temperature ABTS solution to each well.
18. Incubate, with shaking, for 10 to 15 minutes. Remove any bubbles.
19. Stop reaction by adding 20 µl of 10% SDS to each well.
20. Read results on Dynatech MR7000 ELISA reader with test filter at 410 nM and reference filter at 630

nM.

PYK2 BIOASSAY

This assay is used to measure the *in vitro* kinase activity of HA epitope-tagged full length pyk2 (FL.pyk2-HA) in an ELISA assay.

Materials and reagents:

1. Corning 96-well Elisa plates.
2. 12CA5 monoclonal anti-HA antibody (SUGEN, Inc.)
3. PBS (Dulbecco's Phosphate-Buffered Saline (Gibco Catalog # 450-1300EB)
4. TBST Buffer: for 1 L, mix 8.766 g NaCl, 6.057 g TRIS and 1 ml of 0.1% Triton X-100 in approx. 900 ml dH_2O. Adjust pH to 7.2, bring volume to 1 L.
5. Blocking Buffer: for 1 L, mix 100 g 10% BSA, 12.1 g 100 mM TRIS, 58.44 g 1M NaCl and 10 mL of 1% TWEEN-20.
6. FL.pyk2-HA from sf9 cell lysates (SUGEN, Inc.).
7. 4% DMSO in MilliQue H_2O.
8. 10 mM ATP in dH_2O.
9. 1M $MnCl_2$.
10. 1M $MgCl_2$.
11. 1M Dithiothreitol (DTT).
12. 10X Kinase buffer phosphorylation: mix 5.0 ml 1M Hepes (pH 7.5), 0.2 ml 1M $MnCl_2$, 1.0 ml 1 M $MgCl_2$, 1.0 ml 10% Triton X-100 in 2.8 ml dH_2O. Just prior to use, add 0.1 ml 1M DTT.
13. NUNC 96-well V bottom polypropylene plates.
14. 500 mM EDTA in dH_2O.
15. Antibody dilution buffer: for 100 mL, 1 mL 5% BSA/PBS and 1 mL 10% Tween-20 in 88 mL TBS.
16. HRP-conjugated anti-Ptyr PY99), Santa Cruz Biotech Cat. No. SC-7020.
17. ABTS, Moss, Cat. No. ABST-2000.
18. 10% SDS.

Procedure:

1. Coat Corning 96 well ELISA plates with 0.5 µg per

well 12CA5 anti-HA antibody in 100 µl PBS. Store overnight at 4°C.

2. Remove unbound HA antibody from wells by inverting plate. Wash plate with dH_2O. Pat the plate on a paper towel to remove excess liquid.
3. Add 150 µl Blocking Buffer to each well. Incubate, with shaking, for 30 min at room temperature.
4. Wash plate 4x with TBS-T.
5. Dilute lysate in PBS (1.5 µg lysate/100 µl PBS).
6. Add 100 µl of diluted lysate to each well. Shake at room temperature for 1 hr.
7. Wash as in step 4.
8. Add 50 µl of 2X kinase Buffer to ELISA plate containing captured pyk2-HA.
9. Add 25 µL of 400 µM test compound in 4% DMSO to each well. For control wells use 4% DMSO alone.
10. Add 25 µL of 0.5 M EDTA to negative control wells.
11. Add 25 µl of 20 µM ATP to all wells. Incubate, with shaking, for 10 minutes.
12. Stop reaction by adding 25 µl 500 mM EDTA (pH 8.0) to all wells.
13. Wash as in step 4.
14. Add 100 µL HRP conjugated anti-Ptyr diluted 1:6000 in Antibody Dilution Buffer to each well. Incubate, with shaking, for 1 hr. at room temperature.
15. Wash plate 3X with TBST and 1X with PBS.
16. Add 100 µL of ABST solution to each well.
17. If necessary, stop the development reaction by adding 20 µL 10% SDS to each well.
18. Read plate on ELISA reader with test filter at 410 nM and reference filter at 630 nM.

FGFR1 BIOASSAY

This assay is used to measure the *in vitro* kinase activity of FGF1-R in an ELISA assay.

Materials and Reagents:

1. Costar 96-well Elisa plates (Corning Catalog # 3369).
2. Poly(Glu-Tyr) (Sigma Catalog # P0275).
3. PBS (Gibco Catalog # 450-1300EB)
4. 50 mM Hepes Buffer Solution.
5. Blocking Buffer (5% BSA/PBS).
6. Purified GST-FGFR1 (SUGEN, Inc.)
7. Kinase Dilution Buffer.
   Mix 500 µl 1M Hepes (GIBCO), 20 µl 5% BSA/PBS, 10 µl 100mM sodium orthovanadate and 50 µl 5M NaCl.
8. 10mM ATP
9. ATP/$MnCl_2$ phosphorylation mix: mix 20 µL ATP, 400 µL 1M $MnCl_2$ and 9.56 ml dH_2O.
10. NUNC 96-well V bottom polypropylene plates (Applied Scientific Catalog # AS-72092).
11. 0.5M EDTA.
12. 0.05% TBST
    Add 500 µL TWEEN to 1 liter TBS.
13. Rabbit polyclonal anti-phosphotyrosine serum (SUGEN, Inc.).
14. Goat anti-rabbit IgG peroxidase conjugate (Biosource, Catalog # ALI0404).
15. ABTS Solution.
16. ABTS/H_2O_2 solution.

Procedure:

1. Coat Costar 96 well ELISA plates with 1 µg per well Poly(Glu,Tyr) in 100µl PBS. Store overnight at 4° C.
2. Wash coated plates once with PBS.
3. Add 150 µL of 5%BSA/PBS Blocking Buffer to each well. Incubate, with shaking, for 1 hr.room temperature.
4. Wash plate 2x with PBS, then once with 50mM Hepes. Pat plates on a paper towel to remove excess liquid and bubbles.
5. Add 25µL of 0.4 mM test compound in 4% DMSO or 4%

DMSO alone (controls) to plate.

6. Dilute purified GST-FGFR1 in Kinase Dilution Buffer (5 ng kinase/50ul KDB/well).
7. Add 50μL of diluted kinase to each well.
8. Start kinase reaction by adding 25μl/well of freshly prepared ATP/Mn++ (0.4 ml 1M $MnCl_2$, 40 μL 10 mM ATP, 9.56 ml dH_2O), freshly prepared).
9. This is a fast kinase reaction and must be stopped with 25μL of 0.5M EDTA in a manner similar to the addition of ATP.
10. Wash plate 4x with fresh TBST.
11. Make up Antibody Dilution Buffer: Per 50 ml: Mix 5 ml of 5% BSA, 250 μl of 5% milk and 50 μl of 100mM sodium vanadate, bring to final volume with 0.05% TBST.
12. Add 100 μl per well of anti-phosphotyrosine (1:10000 dilution in ADB). Incubate, with shaking for 1 hr. at room temperature.
13. Wash as in step 10.
14. Add 100 μl per well of Biosource Goat anti-rabbit IgG peroxidase conjugate (1:6000 dilution in ADB). Incubate, with shaking for 1 hr. at room temperature.
15. Wash as in step 10 and then with PBS to remove bubbles and excess TWEEN.
16. Add 100 μl of ABTS/H_2O_2 solution to each well.
17. Incubate, with shaking, for 10 to 20 minutes. Remove any bubbles.
18. Read assay on Dynatech MR7000 elisa reader: test filter at 410 nM, reference filtrate 630 nM.

EGFR BIOASSAY

This assay is used to the *in vitro* kinase activity of FGF1-R in an ELISA assay.

Materials and Reagents:

1. Corning 96-well Elisa plates.

2. SUMO1 monoclonal anti-EGFR antibody (SUGEN, Inc.).
3. PBS
4. TBST Buffer
5. Blocking Buffer: for 100 ml, mix 5.0 g Carnation Instant Non-fat Milk® with 100 ml of PBS.
6. A431 cell lysate (SUGEN, Inc.).
7. TBS Buffer:
8. TBS + 10% DMSO: for 1L, mix 1.514 g TRIS, 2.192 g NaCl and 25 ml DMSO; bring to 1 liter total volume with dH_2O.
9. ATP (Adenosine-5'-triphosphate, from Equine muscle, Sigma Cat. No. A-5394), 1.0 mM solution in dH_2O. This reagent should be made up immediately prior to use and kept on ice.
10. 1.0 mM $MnCl_2$.
11. ATP/$MnCl_2$ phosphorylation mix: to make 10 ml, mix 300 µl of 1 mM ATP, 500 µl $MnCl_2$ and 9.2 ml dH_2O. Prepare just prior to use, keep on ice.
12. NUNC 96-well V bottom polypropylene plates.
13. EDTA.
14. Rabbit polyclonal anti-phosphotyrosine serum (SUGEN, Inc.).
15. Goat anti-rabbit IgG peroxidase conjugate (Biosource Cat. No. ALI0404).
16. ABTS.
17. 30% Hydrogen peroxide.
18. ABTS/H_2O_2.
19. 0.2 M HCl.

Procedure:

1. Coat Corning 96 well ELISA plates with 0.5 µg SUMO1 in 100 µl PBS per well, store overnight at 4° C.
2. Remove unbound SUMO1 from wells by inverting plate to remove liquid. Wash 1x with dH_2O. Pat the plate on a paper towel to remove excess liquid.

3. Add 150 µl of Blocking Buffer to each well. Incubate, with shaking, for 30 min. at room temperature.
4. Wash plate 3x with deionized water, then once with TBST. Pat plate on a paper towel to remove excess liquid and bubbles.
5. Dilute lysate in PBS (7 µg lysate/100 µl PBS).
6. Add 100 µl of diluted lysate to each well. Shake at room temperature for 1 hr.
7. Wash plates as in 4, above.
8. Add 120 µl TBS to ELISA plate containing captured EGFR.
9. Dilute test compound 1:10 in TBS, place in well
10. Add 13.5 µl diluted test compound to ELISA plate. To control wells, add 13.5 µl TBS in 10% DMSO.
11. Incubate, with shaking, for 30 minutes at room temperature.
12. Add 15 µl phosphorylation mix to all wells except negative control well. Final well volume should be approximately 150 µl with 3 µM ATP/5 mM $MnCl_2$ final concentration in each well. Incubate with shaking for 5 minutes.
13. Stop reaction by adding 16.5 µl of EDTA solution while shaking. Shake for additional 1 min.
14. Wash 4x with deionized water, 2x with TBST.
15. Add 100 µl anti-phosphotyrosine (1:3000 dilution in TBST) per well. Incubate, with shaking, for 30-45 min. at room temperature.
16. Wash as in 4, above.
17. Add 100 µl Biosource Goat anti-rabbit IgG peroxidase conjugate (1:2000 dilution in TBST) to each well. Incubate with shaking for 30 min. at room temperature.
18. Wash as in 4, above.
19. Add 100 µl of ABTS/H_2O_2 solution to each well.

20. Incubate 5 to 10 minutes with shaking. Remove any bubbles.
21. If necessary, stop reaction by adding 100 µl 0.2 M HCl per well.
22. Read assay on Dynatech MR7000 ELISA reader: test filter at 410 nM, reference filter at 630 nM.

PDGFR BIOASSAY

This assay is used to the *in vitro* kinase activity of FGF1-R in an ELISA assay.

Materials and Reagents:

1. Corning 96-well Elisa plates
2. 28D4C10 monoclonal anti-PDGFR antibody (SUGEN, Inc.).
3. PBS.
4. TBST Buffer.
5. Blocking Buffer (same as for EGFR bioassay).
6. PDGFR-β expressing NIH 3T3 cell lysate (SUGEN, Inc.).
7. TBS Buffer.
8. TBS + 10% DMSO.
9. ATP.
10. $MnCl_2$.
11. Kinase buffer phosphorylation mix: for 10 ml, mix 250 µl 1M TRIS, 200 µl 5M NaCl, 100 µl 1M $MnCl_2$ and 50µl 100 mM Triton X-100 in enough dH_2O to make 10 ml.
12. NUNC 96-well V bottom polypropylene plates.
13. EDTA.
14. Rabbit polyclonal anti-phosphotyrosine serum (SUGEN,Inc.).
15. Goat anti-rabbit IgG peroxidase conjugate (Biosource Cat. No. ALI0404).
16. ABTS.
17. Hydrogen peroxide, 30% solution.
18. ABTS/H_2O_2.
19. 0.2 M HCl.

Procedure:

1. Coat Corning 96 well ELISA plates with 0.5 µg 28D4C10 in 100 µl PBS per well, store overnight at 4° C.
2. Remove unbound 28D4C10 from wells by inverting plate to remove liquid. Wash 1x with dH_2O. Pat the plate on a paper towel to remove excess liquid.
3. Add 150 µl of Blocking Buffer to each well. Incubate for 30 min. at room temperature with shaking.
4. Wash plate 3x with deionized water, then once with TBST. Pat plate on a paper towel to remove excess liquid and bubbles.
5. Dilute lysate in HNTG (10 µg lysate/100 µl HNTG).
6. Add 100 µl of diluted lysate to each well. Shake at room temperature for 60 min.
7. Wash plates as described in Step 4.
8. Add 80 µl working kinase buffer mix to ELISA plate containing captured PDGFR.
9. Dilute test compound 1:10 in TBS in 96-well polypropylene plates.
10. Add 10 µl diluted test compound to ELISA plate. To control wells, add 10 µl TBS + 10% DMSO. Incubate with shaking for 30 minutes at room temperature.
11. Add 10 µl ATP directly to all wells except negative control well (final well volume should be approximately 100 µl with 20 µM ATP in each well.) Incubate 30 minutes with shaking.
12. Stop reaction by adding 10 µl of EDTA solution to each well.
13. Wash 4x with deionized water, twice with TBST.
14. Add 100 µl anti-phosphotyrosine (1:3000 dilution in TBST) per well. Incubate with shaking for 30-45 min. at room temperature.
15. Wash as in Step 4.
16. Add 100 µl Biosource Goat anti-rabbit IgG peroxidase

conjugate (1:2000 dilution in TBST) to each well. Incubate with shaking for 30 min. at room temperature.

17. Wash as in Step 4.
18. Add 100 μl of ABTS/H_2O_2 solution to each well.
19. Incubate 10 to 30 minutes with shaking. Remove any bubbles.
20. If necessary stop reaction with the addition of 100 μl 0.2 M HCl per well.
21. Read assay on Dynatech MR7000 ELISA reader with test filter at 410 nM and reference filter at 630 nM.

CELLULAR HER-2 KINASE ASSAY

This assay is used to measure HER-2 kinase activity in whole cells in an ELISA format.

Materials and Reagents:

1. DMEM (GIBCO Catalog #11965-092).
2. Fetal Bovine Serum (FBS, GIBCO Catalog #16000-044), heat inactivated in a water bath for 30 min. at 56° C
3. Trypsin (GIBCO Catalog #25200-056).
4. L-Glutamine (GIBCO Catalog #25030-081)
5. HEPES (GIBCO Catalog #15630-080).
6. Growth Media
   Mix 500 ml DMEM, 55 ml heat inactivated FBS, 10 ml HEPES and 5.5 ml L-Glutamine.
7. Starve Media
   Mix 500 ml DMEM, 2.5 ml heat inactivated FBS, 10 ml HEPES and 5.5 ml L-Glutamine.
8. PBS.
9. Flat Bottom 96-well Tissue Culture Micro Titer Plates (Corning Catalog # 25860).
10. 15 cm Tissue Culture Dishes (Corning Catalog #08757148).
11. Corning 96-well ELISA Plates.
12. NUNC 96-well V bottom polypropylene plates.
13. Costar Transfer Cartridges for the Transtar 96

(Costar Catalog #7610).

14. SUMO 1: monoclonal anti-EGFR antibody (SUGEN, Inc.).
15. TBST Buffer.
16. Blocking Buffer : 5% Carnation Instant Milk® in PBS.
17. EGF Ligand: EGF-201, Shinko American, Japan. Suspend powder in 100 uL of 10mM HCl. Add 100uL 10mM NaOH. Add 800 uL PBS and transfer to an Eppendorf tube, store at -20°C until ready to use.
18. HNTG Lysis Buffer
    For Stock 5X HNTG, mix 23.83 g Hepes, 43.83 g NaCl, 500 ml glycerol and 100 ml Triton X-100 and enough dH_2O to make 1 L of total solution.
    For 1X HNTG*, mix 2 ml HNTG, 100 μL 0.1M Na_3VO_4, 250 μL 0.2M $Na_4P_2O_7$ and 100 μL EDTA.
19. EDTA.
20. Na_3VO_4. To make stock solution, mix 1.84 g Na_3VO_4 with 90 ml dH_2O. Adjust pH to 10. Boil in microwave for one minute (solution becomes clear). Cool to room temperature. Adjust pH to 10. Repeat heating/cooling cycle until pH remains at 10.
21. 200 mM $Na_4P_2O_7$.
22. Rabbit polyclonal antiserum specific for phosphotyrosine (anti-Ptyr antibody, SUGEN, Inc.).
23. Affinity purified antiserum, goat anti-rabbit IgG antibody, peroxidase conjugate (Biosource Cat # ALI0404).
24. ABTS Solution.
25. 30 % Hydrogen peroxide solution.
26. $ABTS/H_2O_2$.
27. 0.2 M HCl.

<u>Procedure</u>:

1. Coat Corning 96 well ELISA plates with SUMO1 at 1.0 ug per well in PBS, 100 ul final volume/well. Store overnight at 4°C.
2. On day of use, remove coating buffer and wash plate

3 times with dH_2O and once with TBST buffer. All washes in this assay should be done in this manner, unless otherwise specified.

3. Add 100 ul of Blocking Buffer to each well. Incubate plate, with shaking, for 30 min. at room temperature. Just prior to use, wash plate.
4. Use EGFr/HER-2 chimera/3T3-C7 cell line for this assay.
5. Choose dishes having 80-90 % confluence. Collect cells by trypsinization and centrifuge at 1000 rpm at room temperature for 5 min.
6. Resuspend cells in starve medium and count with trypan blue. Viability above 90% is required. Seed cells in starve medium at a density of 2,500 cells per well, 90 ul per well, in a 96 well microtiter plate. Incubate seeded cells overnight at 37° under 5% CO_2.
7. Start the assay two days after seeding.
8. Test compounds are dissolved in 4% DMSO. Samples are then further diluted directly on plates with starve-DMEM. Typically, this dilution will be 1:10 or greater. All wells are then transferred to the cell plate at a further 1:10 dilution (10µl sample and media into 90 µl of starve media. The final DMSO concentration should be 1% or lower. A standard serial dilution may also be used.
9. Incubate under 5% CO_2 at 37°C for 2 hours.
10. Prepare EGF ligand by diluting stock EGF (16.5 uM) in warm DMEM to 150 nM.
11. Prepare fresh HNTG* sufficient for 100 ul per well; place on ice.
12. After 2 hour incubation with test compound, add prepared EGF ligand to cells, 50 ul per well, for a final concentration of 50 nM. Positive control wells receive the same amount of EGF. Negative controls do not receive EGF. Incubate at 37° C for 10

min.

13. Remove test compound, EGF, and DMEM. Wash cells once with PBS.
14. Transfer HNTG* to cells, 100 ul per well. Place on ice for 5 minutes. Meanwhile, remove blocking buffer from ELISA plate and wash.
15. Scrape cells from plate with a micropipettor and homogenize cell material by repeatedly aspirating and dispensing the HNTG* lysis buffer. Transfer lysate to a coated, blocked, washed ELISA plate. Or, use a Costar transfer cartridge to transfer lysate to the plate.
16. Incubate, with shaking, at room temperature for 1 hr.
17. Remove lysate, wash. Transfer freshly diluted anti-Ptyr antibody (1:3000 in TBST) to ELISA plate, 100 ul per well.
18. Incubate, with shaking, at room temperature, for 30 min.
19. Remove anti-Ptyr antibody, wash. Transfer freshly diluted BIOSOURCE antibody to ELISA plate(1:8000 in TBST, 100 ul per well).
20. Incubate, with shaking, at room temperature for 30 min.
21. Remove BIOSOURCE antibody, wash. Transfer freshly prepared ABTS/H_2O_2 solution to ELISA plate, 100 ul per well.
22. Incubate, with shaking, for 5-10 minutes. Remove any bubbles.
23. Stop reaction with the addition of 100ul of 0.2M HCl per well.
24. Read assay on Dynatech MR7000 ELISA reader with test filter set at 410 nM and reference filter at 630 nM.

CDK2/CYCLIN A ASSAY

This assay is used to measure the *in vitro* serine/threonine kinase activity of human cdk2/cyclin A in a

Scintillation Proximity Assay (SPA).

Materials and Reagents.

1. Wallac 96-well polyethylene terephthalate (flexi) plates (Wallac Catalog # 1450-401).
2. Amersham Redivue [$\gamma^{33}P$] ATP (Amersham catalog #AH 9968).
3. Amersham streptavidin coated polyvinyltoluene SPA beads (Amersham catalog #RPNQ0007). The beads should be reconstituted in PBS without magnesium or calcium, at 20 mg/ml.
4. Activated cdk2/cyclin A enzyme complex purified from Sf9 cells (SUGEN, Inc.).
5. Biotinylated peptide substrate (Debtide). Peptide biotin-X-PKTPKKAKKL is dissolved in dH_2O at a concentration of 5 mg/ml.
6. Peptide/ATP Mixture: for 10 ml, mix 9.979 ml dH_2O, 0.00125 ml "cold" ATP, 0.010 ml Debtide and 0.010 ml $\gamma^{33}P$ ATP. The ultimate concentration per well will be 0.5 μM "cold" ATP, 0.1 μg Debtide and 0.2 μCi $\gamma^{33}P$ ATP.
7. Kinase buffer: for 10 ml, mix 8.85 ml dH_2O, 0.625 ml TRIS(pH 7.4), 0.25 ml 1M $MgCl_2$, 0.25 ml 10% NP40 and 0.025 ml 1M DTT, added fresh just prior to use.
8. 10 mM ATP in dH_2O.
9. 1M Tris, pH adjusted to 7.4 with HCl.
10. 1M $MgCl_2$.
11. 1M DTT.
12. PBS (Gibco Catalog # 14190-144).
13. 0.5M EDTA.
14. Stop solution: For 10 ml, mix 9.25 ml PBS, 0.005 ml 100 mM ATP, 0.1 ml 0.5 M EDTA, 0.1 ml 10% Triton X-100 and 1.25 ml of 20 mg/ml SPA beads.

Procedure:

1. Prepare solutions of test compounds at 5x the desired final concentration in 5% DMSO. Add 10 ul to each well. For negative controls, use 10 ul 5%

DMSO alone in wells.

2. Dilute 5 µl of cdk2/cyclin A solution with 2.1 ml 2x kinase buffer.
3. Add 20 ul enzyme to each well.
4. Add 10 µL of 0.5 M EDTA to the negative control wells.
5. To start kinase reaction, add 20 µL of peptide/ATP mixture to each well. Incubate for 1 hr. without shaking.
6. Add 200 µl stop solution to each well.
7. Hold at least 10 min.
8. Spin plate at approx. 2300 rpm for 3-5 min.
9. Count plate using Trilux or similar reader.

MET TRANSPHOSPHORYLATION ASSAY

This assay is used to measure phosphotyrosine levels on a poly(glutamic acid:tyrosine (4:1)) substrate as a means for identifying agonists/antagonists of met transphosphorylation of the substrate.

Materials and Reagents:

1. Corning 96-well Elisa plates, Corning Catalog # 25805-96.
2. Poly(glu, tyr) 4:1, Sigma, Cat. No; P 0275.
3. PBS, Gibco Catalog # 450-1300EB
4. 50 mM HEPES
5. Blocking Buffer: Dissolve 25 g Bovine Serum Albumin, Sigma Cat. No A-7888, in 500 ml PBS, filter through a 4 µm filter.
6. Purified GST fusion protein containing the Met kinase domain, Sugen, Inc.
7. TBST Buffer.
8. 10% aqueous (MilliQue H_2O) DMSO.
9. 10 mM aqueous (dH_2O) Adenosine-5'-triphosphate, Sigma Cat. No. A-5394.
10. 2X Kinase Dilution Buffer: for 100 ml, mix 10 mL 1M

HEPES at pH 7.5 with 0.4 mL 5% BSA/PBS, 0.2 mL 0.1 M sodium orthovanadate and 1 mL 5M sodium chloride in 88.4 mL dH_2O.

11. 4X ATP Reaction Mixture: for 10 mL, mix 0.4 mL 1 M manganese chloride and 0.02 mL 0.1 M ATP in 9.56 mL dH_2O.
12. 4X Negative Controls Mixture: for 10 mL, mix 0.4 mL 1 M manganese chloride in 9.6 mL dH_2O.
13. NUNC 96-well V bottom polypropylene plates, Applied Scientific Catalog # S-72092
14. 500 mM EDTA.
15. Antibody Dilution Buffer: for 100 mL, mix 10 mL 5% BSA/PBS, 0.5 mL 5% Carnation Instant Milk® in PBS and 0.1 mL 0.1 M sodium orthovanadate in 88.4 mL TBST.
16. Rabbit polyclonal antophosphotyrosine antibody, Sugen, Inc.
17. Goat anti-rabbit horseradish peroxidase conjugated antibody, Biosource, Inc.
18. ABTS Solution: for 1 L, mix 19.21 g citric acid, 35.49 g Na_2HPO_4 and 500 mg ABTS with sufficient dH_2O to make 1 L.
19. ABTS/H_2O_2: mix 15 mL ABST solution with 2µL H_2O_2 five minutes before use.
20. 0.2 M HCl

<u>Procedure</u>:

1. Coat ELISA plates with 2 µg Poly(Glu-Tyr) in 100 µL PBS, store overnight at 4 ° C.
2. Block plate with 150 µL of 5% BSA / PBS for 60 min.
3. Wash plate twice with PBS, once with 50 mM Hepes buffer pH 7.4.
4. Add 50 µl of the diluted kinase to all wells. (Purified kinase is diluted with Kinase Dilution Buffer. Final concentration should be 10 ng/well.)
5. Add 25 µL of the test compound (in 4% DMSO) or DMSO alone (4% in dH_2O) for controls to plate.

6. Incubate the kinase/compound mixture for 15 minutes.
7. Add 25 µL of 40 mM $MnCl_2$ to the negative control wells.
8. Add 25 µL ATP/ $MnCl_2$ mixture to the all other wells (except the negative controls). Incubate for 5 min.
9. Add 25 µL 500 mM EDTA to stop reaction.
10. Wash plate 3x with TBST.
11. Add 100 µL rabbit polyclonal anti-Ptyr diluted 1:10,000 in Antibody Dilution Buffer to each well. Incubate, with shaking, at room temperature for one hour.
12. Wash plate 3x with TBST.
13. Dilute Biosource HRP conjugated anti-rabbit antibody 1: 6,000 in Antibody Dilution buffer. Add 100 µL per well and incubate at room temperature, with shaking, for one hour.
14. Wash plate 1X with PBS.
15. Add 100 µl of ABTS/H_2O_2 solution to each well.
16. If necessary, stop the development reaction with the addition of 100 µl of 0.2M HCl per well.
17. Read plate on Dynatech MR7000 elisa reader with the test filter at 410 nM and the reference filter at 630 nM.

IGF-1 TRANSPHOSPHORYLATION ASSAY

This assay is used to measure the phosphotyrosine level in poly(glutamic acid:tyrosine)(4:1) for the identification of agonists/antagonists of gst-IGF-1 transphosphorylation of a substrate.

Materials and Reagents:

1. Corning 96-well Elisa plates.
2. Poly (Glu-tyr) (4:1), Sigma Cat. No. P 0275.
3. PBS, Gibco Catalog # 450-1300EB.
4. 50 mM HEPES
5. TBB Blocking Buffer: for 1 L, mix 100 g BSA, 12.1 gTRIS (pH 7.5), 58.44 g sodium chloride and 10 mL 1%TWEEN-20.

6. Purified GST fusion protein containing the IGF-1 kinase domain (Sugen, Inc.)
7. TBST Buffer: for 1 L, mix 6.057 g Tris, 8.766 g sodium chloride and 0.5 ml TWEEN-20 with enough dH_2O to make 1 liter.
8. 4% DMSO in Milli-Q H_2O.
9. 10 mM ATP in dH_2O.
10. 2X Kinase Dilution Buffer: for 100 mL, mix 10 mL 1 M HEPES (pH 7.5), 0.4 mL 5% BSA in dH_2O, 0.2 mL 0.1 M sodium orthovanadate and 1 mL 5 M sodium chloride with enough dH_2O to make 100 mL.
11. 4X ATP Reaction Mixture: for 10 mL, mix 0.4 mL 1 M $MnCl_2$ and 0.008 mL 0.01 M ATP and 9.56 mL dH_2O.
12.4X Negative Controls Mixture: mix 0.4 mL 1 M manganese chloride in 9.60 mL dH_2O.
13. NUNC 96-well V bottom polypropylene plates.
14. 500 mM EDTA in dH_2O.
15. Antibody Dilution Buffer: for 100 mL, mix 10 mL 5% BSA in PBS, 0.5 mL 5% Carnation Instant Non-fat Milk® in PBS and 0.1 mL 0.1 M sodium orthovanadate in 88.4 mL TBST.
16. Rabbit Polyclonal antiphosphotyrosine antibody, Sugen, Inc.
17. Goat anti-rabbit HRP conjugated antibody, Biosource.
18. ABTS Solution.
20. ABTS/H_2O_2: mix 15 mL ABTS with 2 µL H_2O_2 5 minutes before using.
21. 0.2 M HCl in dH_2O.

Procedure:

1. Coat ELISA plate with 2.0 µg / well Poly(Glu, Tyr) 4:1 (Sigma P0275) in 100 µl PBS. Store plate overnight at 4° C.
2. ash plate once with PBS.
3. Add 100 µl of TBB Blocking Buffer to each well. Incubate plate for 1 hour with shaking at room temperature.

4. Wash plate once with PBS, then twice with 50 mM Hepes buffer pH 7.5.
5. Add 25 μL of test compound in 4% DMSO (obtained by diluting a stock solution of 10 mM test compound in 100% DMSO with dH_2O) to plate.
6. Add 10.0 ng of gst-IGF-1 kinase in 50 μl Kinase Dilution Buffer) to all wells.
7. Start kinase reaction by adding 25μl 4X ATP Reaction Mixture to all test wells and positive control wells. Add 25μl 4X Negative Controls Mixture to all negative control wells. Incubates for 10 minutes with shaking at room temperature.
8. Add 25μl 0.5M EDTA (pH 8.0) to all wells.
9. Wash plate 4x with TBST Buffer.
10. Add rabbit polyclonal anti-phosphotyrosine antisera at a dilution of 1:10,000 in 100μl Antibody Dilution Buffer to all wells. Incubate, with shaking, at room temperature for 1 hour.
11. Wash plate as in step 9.
12. Add 100 μL Biosource anti-rabbit HRP at a dilution of 1:10,000 in Antibody dilution buffer to all wells. Incubate, with shaking, at room temperature for 1 hour.
13. Wash plate as in step 9, follow with one wash with PBS to reduce bubbles and excess Tween-20.
14. Develop by adding 100μl/well $ABTS/H_2O_2$ to each well.
15. After about 5 minutes, read on ELISA reader with test filter at 410 nm and referenced filter at 630 nm.

BRDU INCORPORATION ASSAYS

The following assays use cells engineered to express a selected receptor and then evaluate the effect of a compound of interest on the activity of ligand-induced DNA synthesis by determining BrdU incorporation into the DNA.

The following materials, reagents and procedure are

general to each of the following BrdU incorporation assays. Variances in specific assays are noted.

Materials and Reagents:

1. The appropriate ligand.
2. The appropriate engineered cells.
3. BrdU Labeling Reagent: 10 mM, in PBS (pH 7.4)(Boehringer Mannheim, Germany).
4. FixDenat: fixation solution (ready to use)(Boehringer Mannheim, Germany).
5. Anti-BrdU-POD: mouse monoclonal antibody conjugated with peroxidase (Boehringer Mannheim, Germany).
6. TMB Substrate Solution: tetramethylbenzidine (TMB, Boehringer Mannheim, Germany).
7. PBS Washing Solution : 1X PBS, pH 7.4.
8. Albumin, Bovine (BSA), fraction V powder (Sigma Chemical Co., USA).

General Procedure:

1. Cells are seeded at 8000 cells/well in 10% CS, 2 mM Gln in DMEM, in a 96 well plate. Cells are incubated overnight at 37°C in 5% CO_2.
2. After 24 hours, the cells are washed with PBS, and then are serum-starved in serum free medium (0% CS DMEM with 0.1% BSA) for 24 hours.
3. On day 3, the appropriate ligand and the test compound are added to the cells simultaneously. The negative control wells receive serum free DMEM with 0.1% BSA only; the positive control cells receive the ligand but no test compound. Test compounds are prepared in serum free DMEM with ligand in a 96 well plate, and serially diluted for 7 test concentrations.
4. After 18 hours of ligand activation, diluted BrdU labeling reagent (1:100 in DMEM, 0.1% BSA) is added and the cells are incubated with BrdU (final concentration=10 μM) for 1.5 hours.
5. After incubation with labeling reagent, the medium

is removed by decanting and tapping the inverted plate on a paper towel. FixDenat solution is added (50 µl/well) and the plates are incubated at room temperature for 45 minutes on a plate shaker.

6. The FixDenat solution is thoroughly removed by decanting and tapping the inverted plate on a paper towel. Milk is added (5% dehydrated milk in PBS, 200 µl/well) as a blocking solution and the plate is incubated for 30 minutes at room temperature on a plate shaker.
7. The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:200 dilution in PBS, 1% BSA) is added (50 µl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.
8. The antibody conjugate is thoroughly removed by decanting and rinsing the wells 5 times with PBS, and the plate is dried by inverting and tapping on a paper towel.
9. TMB substrate solution is added (100 µl/well) and incubated for 20 minutes at room temperature on a plate shaker until color development is sufficient for photometric detection.
10. The absorbance of the samples are measured at 410 nm (in "dual wavelength" mode with a filter reading at 490 nm, as a reference wavelength) on a Dynatech ELISA plate reader.

EGF-Induced BrdU Incorporation Assay

Materials and Reagents:

1. Mouse EGF, 201 (Toyobo Co., Ltd., Japan).
2. 3T3/EGFRc7.

EGF-Induced Her-2-driven BrdU Incorporation Assay

Materials and Reagents:

1. Mouse EGF, 201 (Toyobo Co., Ltd., Japan).
2. 3T3/EGFr/Her2/EGFr (EGFr with a Her-2 kinase domain).

EGF-Induced Her-4-driven BrdU Incorporation Assay

Materials and Reagents:

1. Mouse EGF, 201 (Toyobo Co., Ltd., Japan).
2. 3T3/EGFr/Her4/EGFr (EGFr with a Her-4 kinase domain).

PDGF-Induced BrdU Incorporation Assay

Materials and Reagents:

1. Human PDGF B/B (Boehringer Mannheim, Germany).
2. 3T3/EGFRc7.

FGF-Induced BrdU Incorporation Assay

Materials and Reagents:

1. Human FGF2/bFGF (Gibco BRL, USA).
2. 3T3c7/EGFr

IGF1-Induced BrdU Incorporation Assay

Materials and Reagents:

1. Human, recombinant (G511, Promega Corp., USA)
2. 3T3/IGF1r.

Insulin-Induced BrdU Incorporation Assay

Materials and Reagents:

1. Insulin, crystalline, bovine, Zinc (13007, Gibco BRL, USA).
2. 3T3/H25.

HGF-Induced BrdU Incorporation Assay

Materials and Reagents:

1. Recombinant human HGF (Cat. No. 249-HG, R&D Systems, Inc. USA).
2. BxPC-3 cells (ATCC CRL-1687).

Procedure:

1. Cells are seeded at 9000 cells/well in RPMI 10% FBS in a 96 well plate. Cells are incubated overnight at 37°C in 5% CO_2.
2. After 24 hours, the cells are washed with PBS, and then are serum starved in 100 µl serum-free medium (RPMI with 0.1% BSA) for 24 hours.

3. On day 3, 25 µl containing ligand (prepared at 1 µg/ml in RPMI with 0.1% BSA; final HGF conc. is 200 ng/ml) and test compounds are added to the cells. The negative control wells receive 25 µl serum-free RPMI with 0.1% BSA only; the positive control cells receive the ligand (HGF) but no test compound. Test compounds are prepared at 5 times their final concentration in serum-free RPMI with ligand in a 96 well plate, and serially diluted to give 7 test concentrations. Typically, the highest final concentration of test compound is 100 µM, and 1:3 dilutions are used (i.e. final test compound concentration range is 0.137-100 µM).
4. After 18 hours of ligand activation, 12.5 µl of diluted BrdU labeling reagent (1:100 in RPMI, 0.1% BSA) is added to each well and the cells are incubated with BrdU (final concentration is 10 µM) for 1 hour.
5. Same as General Procedure.
6. Same as General Procedure.
7. The blocking solution is removed by decanting and the wells are washed once with PBS. Anti-BrdU-POD solution (1:100 dilution in PBS, 1% BSA) is added (100 µl/well) and the plate is incubated for 90 minutes at room temperature on a plate shaker.
8. Same as General Procedure.
9. Same as General Procedure.
10. Same as General Procedure.

HUV-EC-C Assay

This assay is used to measure a compound's activity against PDGF-R, FGF-R, VEGF, aFGF or Flk-1/KDR, all of which are naturally expressed by HUV-EC cells.

DAY 0

1. Wash and trypsinize HUV-EC-C cells (human umbilical vein endothelial cells, (American Type Culture Collection,

catalogue no. 1730 CRL). Wash with Dulbecco's phosphate-buffered saline (D-PBS, obtained from Gibco BRL, catalogue no. 14190-029) 2 times at about 1 ml/10 cm^2 of tissue culture flask. Trypsinize with 0.05% trypsin-EDTA in non-enzymatic cell dissociation solution (Sigma Chemical Company, catalogue no. C-1544). The 0.05% trypsin is made by diluting 0.25% trypsin/1 mM EDTA (Gibco, catalogue no. 25200-049) in the cell dissociation solution. Trypsinize with about 1 ml/25-30 cm^2 of tissue culture flask for about 5 minutes at 37°C. After cells have detached from the flask, add an equal volume of assay medium and transfer to a 50 ml sterile centrifuge tube (Fisher Scientific, catalogue no. 05-539-6).

2. Wash the cells with about 35 ml assay medium in the 50 ml sterile centrifuge tube by adding the assay medium, centrifuge for 10 minutes at approximately 200x g, aspirate the supernatant, and resuspend with 35 ml D-PBS. Repeat the wash two more times with D-PBS, resuspend the cells in about 1 ml assay medium/15 cm^2 of tissue culture flask. Assay medium consists of F12K medium (Gibco BRL, catalogue no. 21127-014) and 0.5% heat-inactivated fetal bovine serum. Count the cells with a Coulter Counter® (Coulter Electronics, Inc.) and add assay medium to the cells to obtain a concentration of 0.8-1.0 x 10^5 cells/ml.

3. Add cells to 96-well flat-bottom plates at 100 µl/well or 0.8-1.0 x 10^4 cells/well, incubate ~24h at 37°C, 5% CO_2.

DAY 1

1. Make up two-fold test compound titrations in separate 96-well plates, generally 50 µM on down to 0 µM. Use the same assay medium as mentioned in day 0, step 2 above. Titrations are made by adding 90 µl/well of test compound at 200 µM (4X the final well concentration) to the top well of a particular plate column. Since the stock test compound is usually 20 mM in DMSO, the 200 µM drug concentration contains 2% DMSO.

A diluent made up to 2% DMSO in assay medium (F12K + 0.5%

fetal bovine serum) is used as diluent for the test compound titrations in order to dilute the test compound but keep the DMSO concentration constant. Add this diluent to the remaining wells in the column at 60 μl/well. Take 60 μl from the 120 μl of 200 μM test compound dilution in the top well of the column and mix with the 60 μl in the second well of the column. Take 60 μl from this well and mix with the 60 μl in the third well of the column, and so on until two-fold titrations are completed. When the next-to-the-last well is mixed, take 60 μl of the 120 μl in this well and discard it. Leave the last well with 60 μl of DMSO/media diluent as a non-test compound-containing control. Make 9 columns of titrated test compound, enough for triplicate wells each for: (1) VEGF (obtained from Pepro Tech Inc., catalogue no. 100-200, (2) endothelial cell growth factor (ECGF) (also known as acidic fibroblast growth factor, or aFGF) (obtained from Boehringer Mannheim Biochemica, catalogue no. 1439 600), or, (3) human PDGF B/B (1276-956, Boehringer Mannheim, Germany) and assay media control. ECGF comes as a preparation with sodium heparin.

2. Transfer 50 μl/well of the test compound dilutions to the 96-well assay plates containing the $0.8\text{-}1.0\times10^4$ cells/100 μl/well of the HUV-EC-C cells from day 0 and incubate ~2 h at 37° C, 5% CO_2.

3. In triplicate, add 50 μl/well of 80 μg/ml VEGF, 20 ng/ml ECGF, or media control to each test compound condition. As with the test compounds, the growth factor concentrations are 4X the desired final concentration. Use the assay media from day 0 step 2 to make the concentrations of growth factors. Incubate approximately 24 hours at 37°C, 5% CO_2. Each well will have 50 μl test compound dilution, 50 μl growth factor or media, and 100 μl cells, which calculates to 200 μl/well total. Thus the 4X concentrations of test compound and growth factors become 1X once everything has been added to the wells.

DAY 2

1. Add ^{3}H-thymidine (Amersham, catalogue no. TRK-686) at 1 μCi/well (10 μl/well of 100 μCi/ml solution made up in RPMI media + 10% heat-inactivated fetal bovine serum) and incubate ~24 h at 37°C, 5% CO_2. RPMI is obtained from Gibco BRL, catalogue no. 11875-051.

DAY 3

1. Freeze plates overnight at -20°C.

DAY 4

Thaw plates and harvest with a 96-well plate harvester (Tomtec Harvester 96®) onto filter mats (Wallac, catalogue no. 1205-401), read counts on a Wallac Betaplate™ liquid scintillation counter.

TABLE 3 shows the results of biological testing of some exemplary compounds of this invention. The results are reported in terms of IC_{50}, the micromolar (μM) concentration of the compound being tested which causes a 50% change in the activity of the target PKT compared to the activity of the PTK in a control to which no test compound has been added. Specifically, the results shown indicate the concentration of a test compound needed to cause a 50% reduction of the activity of the target PTK. Bioassays which have been or can be used to evaluate compounds are described in detail below.

TABLE 3

Example	bio flkGST IC50 (μM)	bio FGFR1 IC50 (μM)	bio PDGF IC50 (μM)	bio EGF IC50 (μM)	cell EGF IC50 (μM)	Her2 Kinase IC50 (μM)	cdk2spa C50 (μM)	bio pyk2 IC50 (μM)
1	57.68	15.16	>100	>100	>100			>100
2	>100		>100	>100	>100			
3	9.85	9.62	>100	>100	>100			>100
4	3.57	>20	>100	>100	>100	>100		
5	8.3	16.06	>100	>100	>100	>100		
6	4.04		>100	3.26	7.82	2.43		
7	7.74		>100	5.07	9.8	4.24		
8	12.1		>100	51.34	20.08	5.5		
9	0.96		>100	>100	>100	16.38		
10	5.72		>100	94.04	15.86	8.06		

Example	bio flkGST IC50 (μM)	bio FGFR1 IC50 (μM)	bio PDGF IC50 (μM)	bio EGF IC50 (μM)	cell EGF IC50 (μM)	Her2 Kinase IC50 (μM)	cdk2spa C50 (μM)	bio pyk2 IC50 (μM)
11	9.77		>100	>100	>100	>100		
12	>20		21.46	>100		27.73		
13	>20		81.92	8.17		2.66		
14	13.01		42.41	>100		66.02		
15	>20		>100	>100		98.61		
16	>20		98.06	>100		23.32		
17	8.25	2.47	94.35	0.83	11.47	15.94	>10	
18	2.67			2.57	9.23	4.99		
19	7.5			6.86	34.18	8.37		
20	11.53			>100	41.16	8		
21	7.18		>100	40.34		27.69		
22	>20		>100	>100		87.67		
23	>20		>100	36.64		4.05		
24			>100	16.84		5.31		
25	12.55		>100	23.48		7.9		
26	16.03		66.87	34.67		10.04		
27			>100	26.5		3.91		
28	4.5		71.27	53.66		2.67		
29	10.12		>100	26.72		3.98		
30	9.4		>100	18.69		4.1		
31	>50		>100	9.83		47.19		
32	45.74		5.94	>100		>100		
34	>50		>100	>100		>100		
35	>20		>100	80.4		54.14		
36	>20		>100	>100		>100		
37	0.22		3.06	10.78	9.84	1.4		
38	4.17		3.06	6.04	8.97	2.16		
39	3.38		4.69	3.67	14.54	3.53		
40	4.5		7.9	6.52		6.27		
42	0.1		0.12	11.95	74.55	20.43		
43	1.12		8.38	>100	37.33	53.37		
44	<0.05		0.02	20.73	67.46	6.99		
45	1.71		>100	>100	29.95	>100		
46	30.62		6.18	>100	>100	>100		
47	0.08	1.56	0.06	11.42	41.54	8.4	>20	1.05
48	0.006	0.3	<0.78	17.88	21.58	7.93		0.09
49			<0.78	>100	43.86	>100		
50			<0.78	>100	20.34	>100		

Example	bio flkGST IC50 (μM)	bio FGFR1 IC50 (μM)	bio PDGF IC50 (μM)	bio EGF IC50 (μM)	cell EGF IC50 (μM)	Her2 Kinase IC50 (μM)	cdk2spa C50 (μM)	bio pyk2 IC50 (μM)
51	0.006	1.66	0.01	18.1	21.61	23.24	16.69	0.35
52	0.08	1.26	<0.78	12.53	>100	>100	10.66	0.45
53			<0.78	>100	>100	>100		
54	1.98		<0.78	23.88	9.76	7.02		
55	0.27		0.53	6.03	35.99	77.82		
56	2.32		3.19	>100	10.03	7.11		
57	0.06		7.98	>100	9.97	6.94		
58			21.14	>100	>100	>100		
59			<0.78	>100	>100	>100		
60			<0.78	>100	>100	>100		
61			<0.78	>100	>100	>100		
62	8.00		8.32	>100	>100	>100		
63	0.21		<0.78	8.59	>100	>100		
64	0.55		<0.78	30.49	>100	>100		
65	0.37		<0.05	>100	74.36	15.97		
66	<0.05			>100	11.84	2.76		
67	0.39		24.77	31.38	19.79	2.56		
68	1.16		0.03	>100	23.52	34.13		
69	0.3		56.55	>100	97.54	>100		
70	0.09	1.50	0.0030	10.57	6.42	7.99	12.62	0.63
71	15.21		22.5	>100		9.91		
72	6.06		10.54	>100	39.94	9.65		
73	5.95		14.12	>100	39.5	8.59		
74	1.2		0.09	46.75		>100		
75	2.7		61.55	>100		>100		
76	3.33		19.18	5.11		3.01		
77	0.49		25.01	>100		>100		
78	1.94		70.62	9.33		4.25		
79	1.49		>100	27.39		>100		
80	0.13	4.29	0.001	>100		50.19	17.19	0.28
81	0.21		0.18	>100		>100		
82	2.03	7.69	6.88	>100		>100		0.31
83	0.34	0.41	9.46	2.18		86.9		0.008
84	1.38		12.51	67.2		5.86		0.006
85	0.2	0.8	2.59	>100		3.76		
86	1.45	1.3	19.6	41.8		>100		3.58
87	3.27	7.56	6.46	>100		9.1		0.17
88	0.35	1.18	8.06	2.36		>100		0.09

Example	bio flkGST IC50 (μM)	bio FGFR1 IC50 (μM)	bio PDGF IC50 (μM)	bio EGF IC50 (μM)	cell EGF IC50 (μM)	Her2 Kinase IC50 (μM)	cdk2spa C50 (μM)	bio pyk2 IC50 (μM)
89	7.84		47.58	8.53	9.67	15.97		
115	7.3		7.48	>100		>100	0.006	
116	>20		>100	>100		>100	<0.0005	
117	0.91		12.9	>100		>100	0.006	
118	1.93		1.2	>100		>100	0.002	
119	1.38		61.63	>100		>100	<0.0005	

IN VIVO ANIMAL MODELS

XENOGRAFT ANIMAL MODELS

The ability of human tumors to grow as xenografts in athymic mice (*e.g.*, Balb/c, nu/nu) provides a useful *in vivo* model for studying the biological response to therapies for human tumors. Since the first successful xenotransplantation of human tumors into athymic mice, (Rygaard and Povlsen, 1969, Acta Pathol. Microbial. Scand. 77:758-760), many different human tumor cell lines (*e.g.*, mammary, lung, genitourinary, gastro-intestinal, head and neck, glioblastoma, bone, and malignant melanomas) have been transplanted and successfully grown in nude mice. The following assays may be used to determine the level of activity, specificity and effect of the different compounds of the present invention. Three general types of assays are useful for evaluating compounds: cellular/catalytic, cellular/biological and *in vivo*. The object of the cellular/catalytic assays is to determine the effect of a compound on the ability of a TK to phosphorylate tyrosines on a known substrate in a cell. The object of the cellular/biological assays is to determine the effect of a compound on the biological response stimulated by a TK in a cell. The object of the *in vivo* assays is to determine the effect of a compound in an animal model of a particular disorder such as cancer.

Suitable cell lines for subcutaneous xenograft experiments include C6 cells (glioma, ATCC # CCL 107), A375 cells (melanoma, ATCC # CRL 1619), A431 cells (epidermoid

carcinoma, ATCC # CRL 1555), Calu 6 cells (lung, ATCC # HTB 56), PC3 cells (prostate, ATCC # CRL 1435), SKOV3TP5 cells and NIH 3T3 fibroblasts genetically engineered to overexpress EGFR, PDGFR, IGF-1R or any other test kinase. The following protocol can be used to perform xenograft experiments:

Female athymic mice (BALB/c, nu/nu) are obtained from Simonsen Laboratories (Gilroy, CA). All animals are maintained under clean-room conditions in Micro-isolator cages with Alpha-dri bedding. They receive sterile rodent chow and water ad libitum.

Cell lines are grown in appropriate medium (for example, MEM, DMEM, Ham's F10, or Ham's F12 plus 5% - 10% fetal bovine serum (FBS) and 2 mM glutamine (GLN)). All cell culture media, glutamine, and fetal bovine serum are purchased from Gibco Life Technologies (Grand Island, NY) unless otherwise specified. All cells are grown in a humid atmosphere of 90-95% air and 5-10% CO_2 at 37°C. All cell lines are routinely subcultured twice a week and are negative for mycoplasma as determined by the Mycotect method (Gibco).

Cells are harvested at or near confluency with 0.05% Trypsin-EDTA and pelleted at 450 x g for 10 min. Pellets are resuspended in sterile PBS or media (without FBS) to a particular concentration and the cells are implanted into the hindflank of the mice (8 - 10 mice per group, 2 - 10 x 10^6 cells/animal). Tumor growth is measured over 3 to 6 weeks using venier calipers. Tumor volumes are calculated as a product of length x width x height unless otherwise indicated. P values are calculated using the Students t-test. Test compounds in 50 - 100 μL excipient (DMSO, or VPD:D5W) can be delivered by IP injection at different concentrations generally starting at day one after implantation.

TUMOR INVASION MODEL

The following tumor invasion model has been developed and may be used for the evaluation of therapeutic value and efficacy of the compounds identified to selectively inhibit KDR/FLK-1 receptor.

Procedure

8 week old nude mice (female) (Simonsen Inc.) are used as experimental animals. Implantation of tumor cells can be performed in a laminar flow hood. For anesthesia, Xylazine/Ketamine Cocktail (100 mg/kg ketamine and 5 mg/kg Xylazine) are administered intraperitoneally. A midline incision is done to expose the abdominal cavity (approximately 1.5 cm in length) to inject 10^7 tumor cells in a volume of 100 μl medium. The cells are injected either into the duodenal lobe of the pancreas or under the serosa of the colon. The peritoneum and muscles are closed with a 6-0 silk continuous suture and the skin is closed by using wound clips. Animals are observed daily.

Analysis

After 2-6 weeks, depending on gross observations of the animals, the mice are sacrificed, and the local tumor metastases to various organs (lung, liver, brain, stomach, spleen, heart, muscle) are excised and analyzed (measurement of tumor size, grade of invasion, immunochemistry, in situ hybridization determination, etc.).

C-KIT ASSAY

This assay is used to detect the level of c-kit tyrosine phosphorylation.

MO7E (human acute myeloid leukemia) cells were serum starved overnight in 0.1% serum. Cells were pre-treated with the compound (concurrent with serum starvation), prior to ligand stimulation. Cells were stimulated with 250 ng/ml rh-SCF for 15 minutes. Following stimulation, cells were lysed and immunoprecipitated with an anti-c-kit antibody. Phosphotyrosine and protein levels were determined by Western blotting.

MTT PROLIFERATION ASSAY

MO7E cells were serum starved and pre-treated with compound as described for the phosphorylation experiments. Cells were plated @ $4X10^5$ cells/well in a 96 well dish, in 100 μl RPMI + 10% serum. rh-SCF (100 ng/mL) was added and

the plate was incubated for 48 hours. After 48 hours, 10 μl of 5 mg/ml MTT [3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was added and allowed to incubate for 4 hours. Acid isopropanol (100 μl of 0.04N HCl in isopropanol) was added and the optical density was measured at a wavelength of 550 nm.

APOPTOSIS ASSAY

MO7E cells were incubated +/- SCF and +/- compound in 10% FBS with rh-GM-CSF(10ng/mL) and rh-IL-3 (10ng/mL). Samples were assayed at 24 and 48 hours. To measure activated caspase-3, samples were washed with PBS and permeabilized with ice-cold 70% ethanol. The cells were then stained with PE-conjugated polyclonal rabbit anti-active caspase-3 and analyzed by FACS. To measure cleaved PARP, samples were lysed and analyzed by western blotting with an anti-PARP antibody.

Additional assays

Additional assays which may be used to evaluate the compounds of this invention include, without limitation, a bio-flk-1 assay, an EGF receptor-HER2 chimeric receptor assay in whole cells, a bio-src assay, a bio-lck assay and an assay measuring the phosphorylation function of raf. The protocols for each of these assays may be found in U. S. Application Ser. No. 09/099,842, which is incorporated by reference, including any drawings, herein.

Measurement of Cell Toxicity

Therapeutic compounds should be more potent in inhibiting receptor tyrosine kinase activity than in exerting a cytotoxic effect. A measure of the effectiveness and cell toxicity of a compound can be obtained by determining the therapeutic index, i.e., IC_{50}/LD_{50}. IC_{50}, the dose required to achieve 50% inhibition, can be measured using standard techniques such as those described herein. LD_{50}, the dosage which results in 50% toxicity, can also be measured by standard techniques as well (Mossman, 1983, J. Immunol. Methods, 65:55-63), by measuring the amount of LDH released (Korzeniewski and Callewaert, 1983, J. Immunol. Methods,

64:313, Decker and Lohmann-Matthes, 1988, J. Immunol. Methods, 115:61), or by measuring the lethal dose in animal models. Compounds with a large therapeutic index are preferred. The therapeutic index should be greater than 2, preferably at least 10, more preferably at least 50.

B. Example of Cellular Assay Results Using 5-(5-Fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide.

To confirm the potency of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) detected in biochemical assays(*vide infra*), the ability of said compound to inhibit ligand-dependent RTK phosphorylation was evaluated in cell-based assays using NIH-3T3 mouse cells engineered to overexpress Flk-1 or human PDGFRβ. 5-(5-Fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) inhibited VEGF-dependent Flk-1 tyrosine phosphorylation with an IC_{50} value of approximately 0.03 μM. This value is similar to the 0.009 μM K_i value determined for inhibition of Flk-1 by 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) determined in biochemical assays. This indicates that 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) readily penetrates into cells. Consistent with the biochemical data (*vide infra*) indicating that 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) had comparable activity against Flk-1 and PDGFRβ, it was also found that it inhibited PDGF-dependent receptor phosphorylation in cells with an IC_{50} value of approximately 0.03 μM. The ability of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-

diethylamino-ethyl)amide (Compound 80) to inhibit c-kit, a closely related RTK that binds stem cell factor (SCF), was determined using MO7E cells that express this receptor. In these cells, 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) inhibited SCF-dependent c-kit phosphorylation with an IC_{50} value of 0.01-0.1 μM. This compound also inhibited SCF-stimulated c-kit phosphorylation in acute myeloid leukemia (AML) blasts isolated from the peripheral blood of patients.

In addition to testing the ability of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) to inhibit ligand-dependent receptor phosphorylation in cells, its effect on ligand-dependent proliferative response of cells was also examined *in vitro* (*see* Table 4). In these studies, cells quiesced by overnight serum starvation were induced to undergo DNA synthesis upon addition of the appropriate mitogenic ligand. As shown in Table 4, 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) inhibited the PDGF-induced proliferation of NIH-3T3 cells overexpressing PDGFRβ or PDGFRα with IC_{50} values 0.031 and 0.069 μM, respectively, and the SCF-induced proliferation of MO7E cells with an IC_{50} value of 0.007 μM.

TABLE 4

	Biochemical	Cellular IC_{50}	
Receptor	K_i[1] (μM)	Receptor Phosphorylation (μM)	Ligand-dependent Proliferation (μM)
Flk-1/KDR	0.009	0.03[2]	0.004[3]
PDGFRα	0.008	0.03[4]	0.031[4]
PDGFRβ	ND	ND	0.069[5]
FGFR	0.83	ND	0.7[3]
c-kit	ND	0.01-0.1[6]	0.007[6]

ND = Not Determined
[1] Determined using recombinant enzyme
[2] Determined using serum-starved NIH-3T3 cells expressing Flk-1
[3] Determined using serum-starved HUVECs
[4] Determined using serum-starved NIH-3T3 cells expressing PDGFR□
[5] Determined using serum-starved NIH-3T3 cells expressing PDGFR□
[6] Determined using serum-starved MO7E cells

As shown in Table 4, there is a general agreement between the biochemical and cellular activities of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) supporting the conclusion that this compound crosses cellular membranes. Further, it can be concluded that the cellular responses are a result of the activity of compound 80 against the indicated target. In contrast, when tested in the presence of complete growth medium *in vitro*, substantially higher concentrations of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) (>10 μM) were required to inhibit the growth of a variety of human tumor cells (*see* Table 5). This indicates that the compound did not directly inhibit the growth of these cells at concentrations required to inhibit ligand-dependent receptor phosphorylation and cell proliferation.

TABLE 5

Cell Line	Origin	IC_{50} (µM)	LD_{50} (µM)
HT29	Colon carcinoma	10	22
A549	Lung carcinoma	9.5	22
NCI-H460	NSC lung carcinoma	8.9	20
SF767T	Glioma	7.9	14
A431	Epidermoid carcinoma	6.0	18

Briefly, the results shown in Table 5 were obtained by incubating cells for 48 hr in complete growth medium in the presence of serial dilutions 5-(5-Fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide. At the end of the growth period, the relative number of cells was determined. IC_{50} values were calculated as the concentration of compound that inhibited the growth of cells by 50% relative to untreated cells. LD_{50} values were calculated as the concentration of compound that caused a 50% reduction in the number of cells relative to those at the start of the experiment.

A more relevant cell-based assay in which to evaluate the anti-angiogenic potential of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) is the *in vitro* mitogenesis assay using human umbilical vein endothelial cells (HUVECs) as a model system for the endothelial cell proliferation critical to the angiogenic process. In this assay, a mitogenic response, measured as an increase in DNA synthesis, is induced in serum-starved HUVECs upon addition of VEGF or FGF. In these cells, 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) inhibited the VEGF- and FGF-induced mitogenic response in a dose-dependent manner with IC_{50} values of 0.004 µM and 0.7 µM, respectively, when compound was present throughout the 48-hr

assay.

Briefly, the aforementioned results were obtained using Serum-starved HUVECs that were incubated with mitogenic concentrations of VEGF (100 ng/ml) or FGF (30 ng/ml) in the presence of serial dilutions of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) for 24 hrs. The mitogenic response during the following 24 hrs. in the presence of ligand and inhibitor was quantitated by measurement of DNA synthesis based on incorporation of bromodeoxyuridine into cellular DNA.

In separate experiments, compound 80 inhibited the VEGF-dependent phosphorylation of ERK 1/2 (p42/44MAP kinase), an early downstream target of Flk-1/KDR, in a dose-dependent manner. The inhibitory activity of compound 80 was also shown to be long-lasting in this system; inhibiting VEGF-dependent phosphorylation of ERK 1/2 for as long as 48 hours after removal of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) from the medium following a short (2 hr) exposure to micromolar concentrations of the compound.

VEGF has been recognized to be an important survival factor for endothelial cells. Since 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) inhibits the VEGF-dependent mitogenic response of HUVECs, the effect of the compound on HUVEC survival was investigated. In these experiments, cleavage of the caspase 3 substrate poly-ADP-ribosyl polymerase (PARP) was used as a readout for apoptosis. HUVECs cultured in serum-free conditions for 24 hours exhibited substantial levels of PARP cleavage, as detected by the accumulation of the 23 kDa PARP cleavage fragment. This was largely prevented by the addition of VEGF to the cell medium, indicating that VEGF acts as a survival factor in this assay. 5-(5-Fluoro-2-oxo-1,2-dihydroindol-3-

ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) has been shown to inhibit KDR signaling. Accordingly, 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) inhibited VEGF-mediated HUVEC survival in a dose-dependent manner. Thus, these data indicate that compound 80 induced apoptosis in endothelial cells in culture in the presence of VEGF.

C. *In vivo* Efficacy Studies

i. Efficacy Against Established Tumor Xenografts

The *in vivo* efficacy of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) was studied in subcutaneous (SC) xenograft models using human tumor cells implanted into the hindflank region of athymic mice. Following implantation, tumors were allowed to become established to a size of 100-550 mm^3 prior to starting oral treatment with the compound.

Daily oral administration of compound 80 caused a dose-dependent inhibition of A431 tumor growth when treatment was initiated after tumors had grown to a size of 400 mm^3. Statistically significant ($P < 0.05$) inhibition of tumor growth was seen at doses of 40 mg/kg/day (74% inhibition) and 80 mg/kg/day (84% inhibition) (*see* Table 6). In preliminary experiments, a higher (160 mg/kg/day) dose of the compound was not more efficacious against established A431 tumors than the 80 mg/kg/day dose. In addition, mice treated at the 160 mg/kg/day dose of the compound lost body weight, indicating that the higher dose was not as well tolerated. Similar results were obtained in an experiment in which A431 tumors were only allowed to reach 100 mm^3 in size (*see* Table 5). In this second experiment, complete regression of the tumors occurred in six of the eight animals treated at the 80 mg/kg/day for 21 days. In these six animals, the tumors did not regrow during a 110-day observation period following the

end of treatment. In the two animals in which the tumors regrew to a large size (2000-3000 mm^3), the tumors regressed in response to a second round of treatment with compound 80. Importantly, in all efficacy experiments, 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) at 80 mg/kg/day has been well tolerated, even when dosed continuously for more than 100 days.

TABLE 6

Initial Tumor Volume (mm^3)	Compound[1] (mg/kg/day)	% Inhibition (day)	P-Value
400	80	84 (36)	0.001
	40	74 (36)	0.003
	20	51 (36)	0.130
100	80	93 (40)	0.002
	40	75 (40)	0.015
	10	61 (40)	0.059

[1] Compound 80.

Briefly, the results shown in Table 6 were obtained using A431 cells (0.5 x 106 cells/mouse) which were implanted SC into the hindflank region of athymic mice. Daily oral administration of 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) in a Cremophore-based vehicle or vehicle control began when tumors reached the indicated average volume. Tumors were measured using vernier calipers and tumor volume was calculated as the product of length x width x height. P-values were calculated by comparing the size of the tumors for animals that were treated with compound 80 (n=8) to those of animals that were treated with a vehicle (n=16) on the last day of the experiment, using the two-tailed Student's t-test.

The efficacy compound 80 against established human tumors of different origins was determined using Colo205 (colon carcinoma), SF763T (glioma), and NCI-H460 (non-small cell lung carcinoma) xenografts (*see* Table 7). These experiments were conducted using 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) administered orally at 80 mg/kg/day; a dose that was effective and well tolerated.

TABLE 7

	Tumor Type	Initial Tumor Volume (mm^3)	% Inhibition (day)	P-Value
A431[1]	Epidermoid	100	93 (40)	0.002
A431[1]	Epidermoid	400	84 (36)	0.001
Colo205	Colon	370	77 (54)	0.028
NCI-H460	Lung	300	61 (54)	0.003
SF763T	Glioma	550	53 (30)	0.001

[1] Data are from experiment reported in Table 5.

In the abovementioned experiments, compound 80 was administered once daily at 80 mg/kg in a Cremophor-based vehicle once tumors reached the indicated size. Percent inhibition compared to the vehicle-treated control group was calculated at termination of the experiments. P-values were calculated by comparing tumor sizes of the animals that had been treated with the compound to tumor sizes of those animals that had been treated with the vehicle, using the two-tailed Student's t-test.

Although 5-(5-fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1H-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide (Compound 80) inhibited the growth of all the tumor types shown in Table 7, there was a difference in the response of the different xenograft models. Specifically, the growth of NCI-H460 and SF763T tumors was arrested or greatly slowed whereas the Colo205 tumors, like A431 tumors, regressed when treated with 5-(5-Fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide.

In order to determine the molecular basis for the difference in response between xenograft models, the SF763T tumors were studied. Therefore, SF763T tumors, which were less responsive to treatment with 5-(5-Fluoro-2-oxo-1,2-dihydroindol-3-ylidenemethyl)-2,4-dimethyl-1*H*-pyrrole-3-carboxylic acid (2-diethylamino-ethyl)amide, have been evaluated at the molecular level using immunohistological techniques to determine the effect of treatment with the

compound. These studies were initially conducted in this tumor type because SF763T tumors are highly vascularized with microvessels that strongly express the endothelial cell marker CD31 and are hence well suited for studies of tumor microvessel density (MVD). Immunohistological evaluation of SF763T tumors indicated that tumors from treated animals had reduced MVD relative to vehicle-treated controls, consistent with an anti-angiogenic mechanism of action for compound 80; MVD was 24.2 ± 4.1 in animals treated with compound 80, compared to 39.3 ± 5.7 for those that were treated with just the vehicle. As anticipated from the associated tumor growth arrest, a pronounced inhibition of tumor cell proliferation was evident in tumors that were treated with compound 80. These tumors had half the mitotic index of those in vehicle-treated tumors (data not shown). The effect compound 80 on MVD and tumor cell proliferation indicates that the compound has profound anti-angiogenic and anti-tumor effects, even under conditions in which tumors do not regress.

The ability of compound 80 to inhibit PDGFR phosphorylation and subsequent signaling *in vivo* was also evaluated in the SF763T tumors, which express high levels of PDGFRβ. Treatment of the SF763T tumors with compound 80 strongly inhibited PDGFRβ tyrosine phosphorylation in established SF763T tumors. Compound 80 also reduced the levels of phosphorylated (activated) phospholipase C gamma (PLC-γ), an immediate downstream indicator of PDGFR activation. These data demonstrate that oral administration of compound 80 causes a direct effect on target (PDGFR) activity in tumors *in vivo*.

Based on the demonstration that the ability of compound 80 to inhibit VEGF-dependent signaling in HUVECs *in vitro* was long-lasting (*vide supra*), the efficacy of the compound was evaluated when the compound was administered infrequently in the Colo205 tumor model. As shown in Table 8, 80 mg/kg (91% inhibition) and 40 mg/kg (84% inhibition) were efficacious when administered daily, but not when administered twice

weekly. In contrast, a higher dose of compound 80 (160 mg/kg) did inhibit (52% inhibition) the growth of established Colo205 tumors when administered twice weekly, suggesting that this compound can demonstrate efficacy when administered infrequently at a higher dose. It should be noted that dosing regimens may be determined by those with ordinary skill in the art without undue experimentation.

TABLE 8

Dose (mg/kg)	Frequency	% Inhibition	P-Value
160	Twice weekly	52	0.085
	Once weekly	17	NS
80	Daily	91	0.039
	Twice weekly	19	NS
	Once weekly	0	NS
40	Daily	84	0.028
	Twice weekly	36	NS
NS: not significant (P >0.05)			

Briefly, the results shown in Table 8 were obtained using Colo205 cells (0.5 x 10^6 cells/mouse) that had been implanted SC into the hindflank region of athymic mice. Oral administration of compound 80 according to the indicated schedule began when tumors reached 400 mm^3. Tumors were measured using vernier calipers and tumor volume was calculated as the product of length x width x height. P-values were calculated by comparing the size of the tumors for animals that were treated with compound 80 to those of animals that were treated with a vehicle on the last day of the experiment, using the two-tailed Student's t-test.

ii. Efficacy of Compound 80 in a Model of Disseminated Disease

In addition to supporting the sustained growth of solid primary tumors, angiogenesis is also an essential component supporting the development of disseminated disease due to metastasis from the primary tumor. The effect of compound 80 on the development of disseminated disease was examined in the B16-F1 mouse melanoma lung colonization model. In this model, B16-F1 cells inoculated intravenously via the tail vein of athymic mice

colonize the lungs and form tumors. As shown in Table 8, oral administration of compound 80 at 80 mg/kg/day effectively reduced the burden of B16-F1 cells in the lung as evaluated by measurements of total lung weight. These data suggest that compound 80 can inhibit disseminated disease *in vivo*.

TABLE 9

	Lung Weight (g)	% Inhibition	P-Value
Vehicle	0.83 ± 0.07	-	-
Compound[1]	0.41 ± 0.04	50	<0.001

[1] Compound 80

Briefly, the results shown in Table 9 were obtained using athymic mice that had been inoculated with B16-F1 tumor cells ($5x10^5$ cells/mouse) via the tail vein. Mice were treated daily with orally administered compound 80 at 80 mg/kg/day (n=10) or vehicle (n=18) for 24 days after tumor cell inoculation. At the end of the treatment period, the mice were sacrificed and their lungs removed and weighed. Percent inhibition was calculated by comparing the lung weight of those animals that had been treated with compound 80, with the lung weight of the animals that had only been treated with vehicle. P-values were determined using the two-tailed Student's t-test.

D. Examples of Biological Activity.

Examples of the *in vitro* potency of compounds of this invention are shown in Table 2.

CONCLUSION

In studies to investigate the pharmacokinetic characteristics of the compounds of the preferred embodiments of the present invention it has been demonstrated that oral administration of a single dose of said compounds resulted in high oral bioavailability in mice. The good oral bioavailability and linear pharmacokinetics indicate that the compounds of the preferred embodiments of the present have favorable pharmacokinetic characteristics.

In addition, the compounds of the preferred embodiments of the present invention are potent inhibitor of the tyrosine kinase activity of the split-kinase domain RTKs Flk-1/KDR and PDGFR, which are involved in angiogenesis, and the RTK c-kit, a receptor for stem cell factor (SCF), that is involved in certain hematologic cancers. At higher concentrations, the compounds of the preferred embodiments of the present invention also inhibit the tyrosine kinase activity of FGFR-1, a third RTK involved in angiogenesis. Consistent with their biochemical activity, the compounds of the preferred embodiments of the present invention inhibit the ligand-dependent tyrosine phosphorylation of target RTKs and the *in vitro* mitogenic response of human umbilical vein endothelial cells (HUVECs) stimulated with VEGF or FGF, of PDGFR-expressing NIH-3T3 cells stimulated with PDGF, and of MO7E acute myeloid leukemia cells stimulated with SCF . In contrast, the compounds of the preferred embodiments of the present invention do not directly inhibit the proliferation of tumor cells in complete growth medium except at concentrations 2 to 3 orders of magnitude higher than those required to inhibit the ligand-dependent mitogenic responses. In mouse xenograft studies, the compounds of the prefered embodiments of the present invention inhibited the growth of established human tumors of various origins in a dose-dependent manner and at concentrations that were well tolerated even upon extended (>100 days) dosing. At 80 mg/kg/day, the compounds of the preferred embodiments of the present invention induced regression of large established A431 and Colo205 tumors, and caused substantial growth inhibition or stasis of SF763T and NCI-H460 tumors. In mice bearing SF763T tumors, the compounds of the preferred embodiments of the present invention caused reductions in microvessel density, phosphorylation of PDGFR in the tumors, and mitotic index in the tumor cells. At this dose, the compounds of the preferred embodiments of the present invention also inhibited lung colonization by B16-F1 tumor cells in a model of tumor metastasis. Regimen studies

demonstrated that the compounds of the preferred embodiments of the present invention are most efficacious when administered daily. Direct evidence of the anti-angiogenic activity of the compounds of the preferred embodiments of the present invention was detected in SF763T tumors in which microvessel density was reduced. Direct evidence that the compounds of the preferred embodiments of the present invention inhibit PDGFR phosphorylation and signaling *in vivo* was also obtained in SF763T tumors.

Taken together, these data support the notion that orally administered compounds of the preferred embodiments of the present invention are anti-angiogenic agents for the treatment of cancers, including solid tumors and hematological malignancies in which angiogenesis and/or signaling through c-kit are important in the disease pathology.

It will be appreciated that the compounds, methods and pharmaceutical compositions of the present invention are effective in modulating PK activity and therefore are expected to be effective as therapeutic agents against RTK, CTK-, and STK-related disorders.

One skilled in the art would also readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent herein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. For example, if X is described as selected from the group consisting of bromine, chlorine, and iodine, claims for X being bromine and claims for X being bromine and chlorine are fully described.

Other embodiments are within the following claims.